

# Procedure & Checklist - 20 kb Template Preparation Using BluePippin™ Size-Selection System

## Before You Begin

This procedure can be used to prepare size-selected libraries from 5 µg of sheared and concentrated DNA using the BluePippin Size-Selection system. If preparing larger amounts of DNA, scale all the reaction volumes proportionally (e.g., if the input amount of DNA is double the amount set forth in this procedure, then double all the reaction volumes listed in the tables). If a BluePippin Size-Selection system is not available, see the *Procedure & Checklist – Greater Than 10 kb Template Preparation Using AMPure® PB Beads*. Note that size selection using the AMPure PB beads does not completely remove all short SMRTbell templates. As a result, the average subread lengths may be shorter.

Consistent and efficient recovery of your sample is critical to successful SMRTbell® template preparation. If using this protocol for the first time, we strongly recommend that you process a control sample first. Using the DNA shearing methods and subsequent AMPure PB bead purification steps described below, you should recover more than 80% of your input DNA (by mass) after AMPure PB bead purification.

Insert Size Target	Insert Size Range	Sheared and Concentrated DNA Amount	Ligation
10 kb to 20 kb	15 kb to 20 kb (size-selected using BluePippin system)	5 µg	Blunt

## Evaluate Genomic DNA Quality

We recommend using a Pulsed Field Electrophoresis system for evaluating gDNA quality. There are two commercially available systems capable of resolving HMW DNA fragments and smears.

- Bio-Rad® CHEF Mapper® XA Pulsed Field Electrophoresis system. The procedure is available [here](#).
- Sage Science's Pippin Pulse Electrophoresis Power Supply. The procedure is available [here](#).

Alternatively, Advanced Analytical Technologies, Inc. FEMTO Pulse is an automated pulsed-field capillary electrophoresis instrument for evaluating the integrity of genomic DNA with a run time of approximately 1.5 hours.

## Fragment and Concentrate DNA

Use a Covaris® g-TUBE® device to shear your DNA sample. General recommendations for use of g-TUBES can be found in the g-TUBE user manual available for download from the Covaris website (see <http://covarisinc.com/products/g-tube/>). Note that although Covaris provides recommendations for shearing gDNA to 20 kb, these parameters result in sheared gDNA with average insert sizes of less than 20 kb, and are **NOT** suitable for use in this protocol. To generate sheared gDNA with average insert sizes >20 kb, you **MUST** follow the specific instructions provided here:

1. Dilute DNA to 200-300 ng/µl in Elution Buffer (EB). The sample volume may range from 20-100 µL.
2. Shear at 5500 rpm (2029 x g) for 2 minutes in an Eppendorf® MiniSpin plus.
3. Check for any residual sample remaining in the upper chamber. If present, re-spin for another 2 minutes. Continue spinning until all the sample has passed through the orifice.
4. Invert and spin at 5500 rpm (2029 x g) until all the sample has passed through the orifice.
5. Recover your sample into a 1.5 mL LoBind microcentrifuge tube.

Depending upon the quality of your sample, approximately 20% sample loss is to be expected as a result of the shearing and concentration process. Therefore, be sure to have sufficient amounts of starting DNA in order to have at least 5 µg of sheared and concentrated DNA (140 ng/µL) for the subsequent repair steps.






STEP	✓	Concentrate DNA	Notes
1		<p>Add <b>0.45X</b> volume of AMPure<sup>®</sup> PB magnetic beads.</p> <p>_____ μL of sample X <b>0.45X</b> = _____ μL of beads</p> <p>Note that the beads must be brought to room temperature before use and all AMPure PB bead purification steps should be performed at room temperature.</p> <p>Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process.</p>	
2		Mix bead/DNA solution thoroughly by tapping the tube gently. Do not pipet to mix.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by gentle end-over-end rotation for 15-20 minutes at room temperature. We recommend using a tube rotator.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear.	
7		<p>With the tube still on the magnetic bead rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet.</p> <p>If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.</p>	
8		<p>Wash beads with freshly prepared 70% ethanol.</p> <p>Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.</p> <ul style="list-style-type: none"> <li>– Do not remove the tube from the magnetic rack.</li> <li>– Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds.</li> <li>– Do not disturb the bead pellet.</li> <li>– After 30 seconds, pipette and discard the 70% ethanol.</li> </ul>	
9		Repeat <a href="#">step 8</a> above.	
10		<p>Remove residual 70% ethanol.</p> <ul style="list-style-type: none"> <li>– Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the <del>tube</del>.</li> <li>– Place the tube back on magnetic bead rack.</li> <li>– Let beads separate fully.</li> <li>– Pipette off any remaining 70% ethanol.</li> </ul>	
11		Check for any remaining droplets in the tube. If droplets are present, repeat <a href="#">step 10</a> .	

STEP	✓	Concentrate DNA	Notes
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 to 60 seconds.	
13		<p>For 5 µg of input sheared gDNA, elute in <b>37 µL</b> Elution buffer. If you started with more than 5 µg input sheared gDNA, scale volume of EB proportionally (i.e., for 6-10 µg of DNA, elute in 72 µL EB).</p> <p>Add the Pacific Biosciences® Elution Buffer volume to your beads. Tap the tube with finger gently to mix, until beads are uniformly resuspended. <u>Do not pipet to mix.</u></p> <ul style="list-style-type: none"> <li>– Elute the DNA by letting the mix stand at room temperature for 2 minutes.</li> <li>– Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.</li> <li>– Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 mL Lo-Bind tube.</li> <li>– Discard the beads.</li> </ul>	
14		<p>Verify your DNA amount and concentration using a Qubit quantitation platform.</p> <ul style="list-style-type: none"> <li>– Measure the DNA concentration using a Qubit fluorometer.</li> <li>– Using 1 µL of the eluted sample, make a 1:10 dilution in EB.</li> <li>– Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit dsDNA HS Assay kit according to the manufacturer's recommendations.</li> </ul> <p>Yield up to this point should be <b>80%</b>. The remaining 9 µL of 1:10 diluted sample may be used for QC by pulsed-field gel electrophoresis or pulsed-field capillary electrophoresis.</p>	
15		The sheared DNA can be stored for up to 24 hours at 4°C or at -20°C for longer duration.	
16		Actual recovery per µL and total available sample material: _____	

## ExoVII Pre-Treatment of DNA

Use the following table to remove single-stranded ends from 5 µg of sheared gDNA. If starting with more than 5 µg of sheared gDNA, scale the reaction volumes proportionally (i.e., for a mass between 6 - 10 µg of DNA scale the total volume to 96 µL).


1. In a LoBind microcentrifuge tube, add the following reagents:

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
Sheared DNA (5 µg)			36.0 µL			
DNA Damage Repair Buffer		10 X	5.0 µL	1 X		
NAD <sup>+</sup>		100 X	0.5 µL	1 X		
ATP high		10 mM	5.0 µL	1 mM		
dNTP		10 mM	0.5 µL	0.1 mM		
ExoVII		10 U/µL	1.0 µL	0.2 U/µL		
Total Volume			48.0 µL			

2. Mix the reaction well by gently tapping the tube.
3. Spin down contents of tube with a quick spin in a microfuge.
4. Incubate at 37°C for 15 minutes, then return the reaction to 4°C.

## Repair DNA Damage


Use the following table to prepare your reaction. For more than 5 µg input DNA, scale all reactions volumes proportionally.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (ExoVII treated)			48.0 µL			
DNA Damage Repair Mix		25 X	2.0 µL	1X		
Total Volume			50.0 µL			

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 37°C for 20-60 minutes, return the reaction to 4°C for 1 to 5 minutes.

## Repair Ends

Use the following table to prepare your reaction then purify the DNA. For more than 5 µg input DNA, scale all reactions volumes proportionally.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (Damage Repaired)			50.0 µL			
End Repair Mix		20 X	2.5 µL	1X		
Total Volume			52.5 µL			

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 25°C for 5-10 minutes, return the reaction to 4°C. Proceed to the next step.

STEP	✓	Purify DNA	Notes
1		Add <b>0.45X</b> volume of AMPure PB beads to the End-Repair reaction.	
2		Mix the bead/DNA solution thoroughly by gently tapping the tube. Do not pipet to mix.	
3		Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.	
4		Allow the DNA to bind to beads by gentle rotation for 15-20 minutes at room temperature. We recommend using a tube rotator.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		<p>Wash beads with freshly prepared 70% ethanol.</p> <p>Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.</p> <ul style="list-style-type: none"> <li>– Do not remove the tube from the magnetic rack.</li> <li>– Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads.</li> <li>– Do not disturb the bead pellet.</li> <li>– After 30 seconds, pipette and discard the 70% ethanol.</li> </ul>	
9		Repeat <a href="#">step 8</a> above.	
10		<p>Remove residual 70% ethanol.</p> <ul style="list-style-type: none"> <li>– Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.</li> <li>– Place the tube back on magnetic bead rack.</li> <li>– Pipette off any remaining 70% ethanol.</li> </ul>	
11		Check for any remaining droplets in the tube. If droplets are present, repeat <a href="#">step 10</a> .	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 to 60 seconds.	
13		<p>For 5 µg of input DNA, elute in <b>23 µL</b> Elution buffer. If you started with more than 5 µg input sheared DNA, scale volume of EB proportionally (i.e., for 6-10 µg of DNA, elute in 46 µL EB).</p> <p>Add the Pacific Biosciences® Elution Buffer volume to your beads. Tap the tube with finger gently to mix. <u>Do not pipet to mix.</u></p> <ul style="list-style-type: none"> <li>– Elute the DNA by letting the mix stand at room temperature for 2 minutes.</li> <li>– Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.</li> <li>– Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 ml Lo-Bind tube.</li> <li>– Discard the beads.</li> </ul>	

<b>14</b>	<p>Optional: Verify your DNA amount and concentration using a Qubit quantitation platform.</p> <ul style="list-style-type: none"> <li>– Measure the DNA concentration using a Qubit fluorometer.</li> <li>– Using 1 <math>\mu</math>L of the eluted sample, make a 1:10 dilution in EB.</li> <li>– Use 1 <math>\mu</math>L of this 1:10 dilution to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer's recommendations.</li> <li>– The remaining 9 <math>\mu</math>L of 1:10 diluted sample may be used for QC by pulsed-field gel electrophoresis or pulse-field capillary electrophoresis.</li> </ul>	
<b>15</b>	The End-Repaired DNA can be stored overnight at 4°C or at -20°C for longer duration.	
<b>16</b>	Actual recovery per $\mu$ L and total available sample material: _____	

## Prepare Blunt Ligation Reaction

Use the following table to prepare your reaction, adding the components below in the order listed. Be sure to mix DNA and adapter BEFORE adding ligase. For more than 5 µg input DNA, scale all reaction volumes proportionally.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (End Repaired)			23.0 µL			
Blunt Adapter (20 µM)	●	20 µM	10.0 µL	5 µM		
Mix before proceeding						
Template Prep Buffer	○	10 X	4.0 µL	1X		
ATP low	●	1 mM	2.0 µL	0.05 mM		
Mix before proceeding						
Ligase	●	30 U/µL	1.0 µL	0.75 U/µL		
Total Volume			40.0 µL			

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 25°C for 15 – 60 minutes. Ligation reactions can be left at 25 °C overnight.
4. Incubate at 65°C for 10 minutes to inactivate the ligase, then return the reaction to 4°C.

## ExoIII/VII Digestion to Remove Failed Ligation Products

Use the following table to prepare your reaction. For more than 5 µg input DNA, scale all reaction volumes proportionally.

Reagent	Tube Cap Color	Stock Conc.	✓	Volume
Ligated DNA				40.0 µL
Mix reaction well by pipetting				
ExoIII	●	100.0 U/µL		1.0 µL
ExoVII	●	10.0 U/µL		1.0 µL
Total Volume				42.0 µL

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 37°C for 1 hour, then return the reaction to 4°C.
4. You must immediately proceed with AMPure PB bead purification after this step.



## Purify SMRTbell Templates with 0.45X AMPure PB Beads

STEP	✓	Purify SMRTbell Templates	Notes
1		Add <b>0.45X</b> volume of AMPure PB beads to the exonuclease-treated DNA.	
2		Mix the bead/DNA solution thoroughly by tapping the tube gently. Do not pipet to mix.	
3		Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.	
4		Allow the DNA to bind to beads by gentle rotation for 15-20 minutes at room temperature. We recommend using a tube rotator.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		<p>Wash beads with freshly prepared 70% ethanol.</p> <p>Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.</p> <ul style="list-style-type: none"> <li>– Do not remove the tube from the magnetic rack.</li> <li>– Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads.</li> <li>– Do not disturb the bead pellet.</li> <li>– After 30 seconds, pipette and discard the 70% ethanol.</li> </ul>	
9		Repeat <a href="#">step 8</a> above.	
10		<p>Remove residual 70% ethanol.</p> <ul style="list-style-type: none"> <li>– Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.</li> <li>– Place the tube back on magnetic bead rack.</li> <li>– Pipette off any remaining 70% ethanol.</li> </ul>	
11		Check for any remaining droplets in the tube. If droplets are present, repeat <a href="#">step 10</a> .	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 to 60 seconds.	
13		<p>For up to 10 µg input non-selected library, elute in <b>31 µL</b> Elution buffer. If you size select more than 10 µg of SMRTbell library, scale volume proportionally.</p> <p>Add the Pacific Biosciences® Elution Buffer volume to your beads. Tap the tube with finger gently to mix. <u>Do not pipet to mix.</u></p> <ul style="list-style-type: none"> <li>– Elute the DNA by letting the mix stand at room temperature for 2 minutes</li> <li>– Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.</li> <li>– Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 ml Lo-Bind tube.</li> <li>– Discard the beads.</li> </ul>	

<b>14</b>	<p>Verify your DNA amount and concentration using a Qubit quantitation platform.</p> <ul style="list-style-type: none"> <li>- Measure the DNA concentration using a Qubit fluorometer.</li> <li>- Using 1 <math>\mu</math>L of the eluted sample, make a 1:10 dilution in EB.</li> <li>- Use 1 <math>\mu</math>L of this 1:10 dilution to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer's recommendations.</li> </ul> <p>Yield up to this point should be 40-60%. The remaining 9 <math>\mu</math>L of 1:10 diluted sample may be used for QC by pulsed-field gel electrophoresis or pulse-field capillary electrophoresis.</p>	
<b>16</b>	<p>Proceed with size selection after AMPure PB Bead purification of exonuclease-treated libraries. Otherwise, samples may be stored at -20°C at this point.</p>	
<b>17</b>	<p>Actual recovery per <math>\mu</math>L and total available sample material: _____</p>	

## Size-Selection Using the BluePippin System

Follow the general instructions in the BluePippin User Manual and User Guides (see [www.sagescience.com](http://www.sagescience.com)), and the specific recommendations below for >15 kb or >20 kb size selection of the SMRTbell templates. Before performing this procedure, ensure that your BluePippin software is up-to-date by checking Sage's website.

It is highly recommended to perform qualitative and quantitative analysis using pulsed-field gel electrophoresis or pulsed-field capillary electrophoresis before size selection. This allows you to choose the appropriate "BPstart" value for Blue Pippin size selection. Choosing the "BPstart" value cutoff without knowing size distribution of SMRTbell Templates might lead to significant sample loss.

If average library insert sizes as determined by pulsed-field gel electrophoresis are less than 15 kb, you may perform >6 kb to >10 kb size-selection using the "0.75%DF Marker S1 high-pass 6-10kb v3" cassette definition file instead. Refer to the High Pass User Guide for SMRTbell Templates available on Sage's website (see [www.sagescience.com](http://www.sagescience.com)) for more information.

STEP	✓	Prepare DNA Samples for Each Lane	Notes
1		Prepare up to 5 µg SMRTbell templates in a final volume of 30 µL Elution Buffer for each lane. Size selection using this protocol can be aggressive and if not cautious, recovery may be impacted.	
2		Bring the Loading Solution to room temperature, then add 10 µL of the Loading Solution to the 30 µL DNA sample. For multiple lanes, scale volumes proportionally. The Loading Solution is viscous so pipet slowly to ensure complete transfer into the DNA sample. <ul style="list-style-type: none"> <li>– Mix by gentle pipetting; do not vortex.</li> <li>– Spin briefly to collect the contents at the bottom of the tube.</li> </ul>	
3		Follow the manufacturer's recommendations to set up a run protocol. <ul style="list-style-type: none"> <li>– When setting up the run protocol, select the "0.75%DF Marker S1 high-pass 15-20kb" cassette definition file.</li> <li>– Using the "Range" selection mode, enter the desired "BPstart" value of 15000 or 20000 bp. A "BP End" value of 50000 bp should automatically appear. Be sure to assign a marker lane.</li> </ul>	
4		Load samples and start the run. Be sure to include the S1 marker in the correct lane. Typical run times are ~5.5 hours.	
5		To maximize recovery of eluted DNA, wait at least 30 minutes after the run terminates before removing the sample from the elution chamber. <ul style="list-style-type: none"> <li>– Collect the eluate into a 1.5 mL DNA LoBind tube.</li> <li>– Wash elution well with 40 µL of Sage Science's 0.1% Tween-20 Wash Solution, and add wash to eluted sample. Washing the elution well may increase yield 10-20%.</li> </ul>	
6		After size selection with the BluePippin System, you have the option to treat the size selected library with DNA Damage Repair Enzymes. Note that we recommend performing the DNA Damage Repair step. For DNA Damage Repair of size selected libraries, proceed to "Concentrate Size-Selected SMRTbell Templates with 1X AMPure PB Beads for DNA Damage Repair" in the next section. If DNA Damage Repair is <b>not</b> required, proceed to "Purify SMRTbell Templates with 1X AMPure PB Beads" on the last page.	






## Concentrate Size-Selected SMRTbell Templates with 1X AMPure PB Beads for DNA Damage Repair

STEP	✓	Purify DNA	Notes
1		Add <b>1X</b> volume of AMPure PB beads to the size selected library.	
2		Mix the bead/DNA solution thoroughly by gently tapping the tube. Do not pipet to mix.	
3		Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.	
4		Allow the DNA to bind to beads by gentle rotation for 15-20 minutes at room temperature. We recommend using a tube rotator.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		<p>Wash beads with freshly prepared 70% ethanol.</p> <p>Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.</p> <ul style="list-style-type: none"> <li>– Do not remove the tube from the magnetic rack.</li> <li>– Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads.</li> <li>– Do not disturb the bead pellet.</li> <li>– After 30 seconds, pipette and discard the 70% ethanol.</li> </ul>	
9		Repeat <a href="#">step 8</a> above.	
10		<p>Remove residual 70% ethanol.</p> <ul style="list-style-type: none"> <li>– Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.</li> <li>– Place the tube back on magnetic bead rack.</li> <li>– Pipette off any remaining 70% ethanol.</li> </ul>	
11		Check for any remaining droplets in the tube. If droplets are present, repeat <a href="#">step 10</a> .	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 to 60 seconds.	
13		<p>Elute in <b>38 µL</b> Elution buffer.</p> <p>Add the Pacific Biosciences Elution Buffer volume to your beads. Tap the tube with finger gently to mix. <u>Do not pipet to mix.</u></p> <ul style="list-style-type: none"> <li>– Elute the DNA by letting the mix stand at room temperature for 2 minutes.</li> <li>– Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.</li> <li>– Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 ml Lo-Bind tube.</li> <li>– Discard the beads.</li> </ul>	

<b>14</b>	<p>Verify your DNA amount and concentration using a Qubit quantitation platform.</p> <ul style="list-style-type: none"> <li>– Using 1 <math>\mu\text{L}</math> of the purified sample, make a 1:10 dilution in EB.</li> <li>– Use 1 <math>\mu\text{L}</math> of this 1:10 dilution to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer's recommendations.</li> </ul> <p>The remaining 9 <math>\mu\text{L}</math> of 1:10 diluted sample may be used for QC by pulsed-field gel electrophoresis or pulse-field capillary electrophoresis</p>	
<b>15</b>	AMPure PB bead purified, size-selected libraries may be stored at $-20^{\circ}\text{C}$ .	

## Repair DNA Damage After Size-Selection

For up to 5  $\mu\text{g}$  of size selected DNA, use a reaction volume of 50  $\mu\text{L}$ . If starting with more than 5  $\mu\text{g}$  of size-selected template, scale reaction volumes proportionally (i.e., for up to 10  $\mu\text{g}$  of size selected DNA use a 100  $\mu\text{L}$  reaction volume).

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.		Notes
Size-selected DNA	–		37.0 $\mu\text{L}$	–		
DNA Damage Repair Buffer		10 X	5.0 $\mu\text{L}$	1 X		
NAD+		100 X	0.5 $\mu\text{L}$	1 X		
ATP high		10 mM	5.0 $\mu\text{L}$	1 mM		
dNTP		10 mM	0.5 $\mu\text{L}$	0.1 mM		
DNA Damage Repair Mix		25 X	2.0 $\mu\text{L}$	1X		
Total Volume			50.0 $\mu\text{L}$	–		

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at  $37^{\circ}\text{C}$  for 60 minutes.
4. Proceed to the final 1X AMPure PB bead purification step in the next section.

## Purify SMRTbell Templates with 1X AMPure PB Beads

STEP	Purify DNA	Notes
1	Add <b>1X</b> volume of AMPure PB beads to the size selected library.	
2	Mix the bead/DNA solution thoroughly by gently tapping the tube.	
3	Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.	
4	Allow the DNA to bind to beads by gentle rotation for 15-20 minutes at room temperature. We recommend using a tube rotator.	
5	Spin down the tube (for 1 second) to collect beads.	
6	Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7	Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8	<p>Wash beads with freshly prepared 70% ethanol.</p> <p>Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.</p> <ul style="list-style-type: none"> <li>– Do not remove the tube from the magnetic rack.</li> <li>– Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads.</li> <li>– Do not disturb the bead pellet.</li> <li>– After 30 seconds, pipette and discard the 70% ethanol.</li> </ul>	
9	Repeat <a href="#">step 8</a> .	
10	<p>Remove residual 70% ethanol.</p> <ul style="list-style-type: none"> <li>– Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.</li> <li>– Place the tube back on magnetic bead rack.</li> <li>– Pipette off any remaining 70% ethanol.</li> </ul>	
11	Check for any remaining droplets in the tube. If droplets are present, repeat <a href="#">step 10</a> .	
12	Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30-60 seconds.	
13	<p>For up to 5 µg size-selected library, elute in <b>10 µL</b> Elution buffer.</p> <p>For more than 5 µg of SMRTbell template, scale volume of EB proportionally (i.e., for up to 10 µg of input DNA, elute in 20 µL EB).</p> <p>Add the Pacific Biosciences® Elution Buffer volume to your beads. Tap the tube with finger gently to mix. <u>Do not pipet to mix.</u></p> <ul style="list-style-type: none"> <li>– Elute the DNA by letting the mix stand at room temperature for 2 minutes</li> <li>– Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.</li> <li>– Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 ml Lo-Bind tube.</li> <li>– Discard the beads.</li> </ul>	

14		Verify your DNA amount and concentration using a Qubit quantitation platform. <ul style="list-style-type: none"> <li>– Using 1 µL of the purified sample, make a 1:10 dilution in EB.</li> <li>– Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer’s recommendations.</li> <li>– The remaining 9 µL of 1:10 diluted sample may be used for QC by pulsed field gel electrophoresis.</li> </ul>	
15		AMPure PB bead purified, size-selected libraries may be stored at -20C.	

## Primer Annealing, Polymerase Binding and Sequencing

Please refer to the Binding Calculator and SMRT Link Sample Setup for RSII and Sequel, respectively.

Revision History (Description)	Version	Date
Removed DNA Damage Repair requirement. Provided elution volume (page 3). On page 11, added step 6 which provides size-selection option. Added DNA Damage Repair after size-selection option. On page 14, added purification step. On page 15, removed specifics related to annealing, binding, and sequencing, and referred to Binding Calculator and SMRT Link Sample Setup.	09	January 2018
ONLY CHANGE IS TO REVISION HISTORY BOX FROM VERSION 09: Concentrate DNA (page 3/step13): Provided Elution volume (37 µL). BluePippin Size Selection (page 11): Added step 6 which provides DNA Damage Repair option after size selection. On page 15, removed specifics related to annealing, binding, and sequencing and referred to Binding Calculator and SMRT Link Sample Setup.	10	January 2018